Focal Points

Application Note FP-121

UVP BioImaging Systems

Understanding and Detection of Green Fluorescent Protein (GFP) and Their Variants

Introduction

Green fluorescent proteins (GFPs) are presently attracting tremendous interest as the first general method to create strong visible fluorescence by purely molecular biological means. GFPs are used as reporters of gene expression, tracers of cell lineage, and as fusion tags to monitor protein localization within living cells. Prior to re-engineering and mutagenesis, the GFP originally cloned from the jellyfish *Aequorea victoria* had been plagued by deficiencies such as low brightness, a significant delay between protein synthesis and fluorescence development. Overcoming these shortfalls, the new variants have shifted excitation and emission wavelengths, creating different colors as well as applications.

Structure

Native GFP is a protein of 238 amino acids in length (27 kDa) with cylindrical symmetry in 11 strands making up a β-barrel (*Figure 1*). The chromophore is a p-hydroxybenzylideneimid-azolinone formed from residues 65-67, which are Ser-Tyr-Gly in the native protein. Chomophore: First, GFP folds into a nearly native conformation, then the imidazolinone is formed by nucleophilic attack of the amide of Gly67 on the carbonyl of residue 65, followed by dehydration. Finally, molecular oxygen dehydrogenates the o~p bond of residue 66 to put its aromatic group into conjugation with the imidazolinone. Only at this stage does the chromophore acquire visible absorbance and fluorescence. GFP is a stable molecule in that up to 10 different site modifications along the protein sequence has resulted in a stable chromophore. Through mutagenesis and gene shuffling researchers have produced genetic variants with differing spectral properties.

Figure 1. β-barrel structure.

GFP Variants

Generally there are a number of variants used for labeling; EBFP, ECFP, EGFP, EYFP and DsRED (*Figure 2*).

There are a number of red shifted variants of GFP (*Table 1*); the "red-shift" refers to a shift of the excitation peak from the wild type 395 nm towards the red of 488 – 505 nanometers (nm). The emission spectra for these new variants remain essentially unaffected (507- 511 nm). Some of the reasoning for the shift was to allow existing filter sets used for Fluorescein (FITC) detection to be utilized. This does allow for some double labeling by selectively exciting wild type and red shifted GFP while maintaining similar emission spectra. Two common red shifted GFPs are S65T and EGFP.

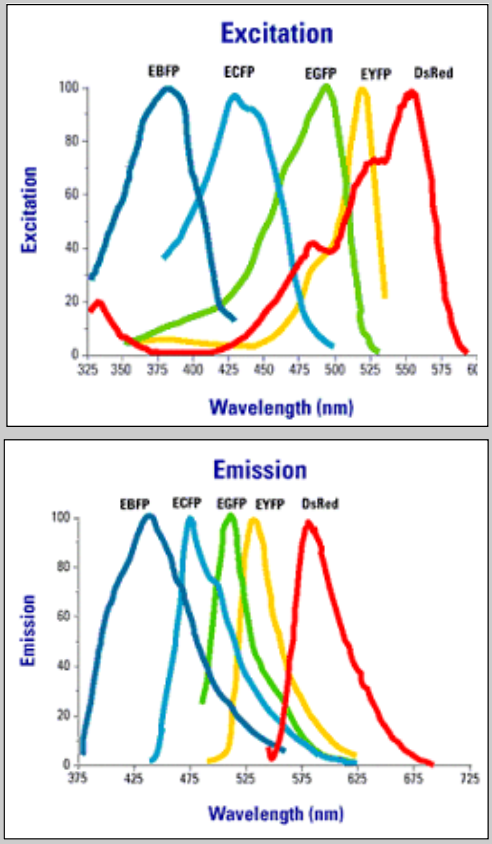

Figure 2. Courtesy of Clontech Laboratories, Palo Alto, CA

Table 1 Common GFP Variants

GFP Variant	*Excitation max (nm)*	*Emission max (nm)*	*Extinction Coefficient ($cm^{-1}M^{-1}$)*
WtGFP	395	509	27,600
	470	509	12,000
EGFP	488	509	61,000
GFP-S65T	489	509	56,000
GFPuv	395	509	27,000
Y66H	382	459	25,000
Stemmer	397	509	27,000
	475	509	12,000
EBFP	380	440	31,000
EYFP	500	527	84,000
	475	509	12,000
ECFP	434	477	26,000
DsRed	558	683	22,500

Fluorescence Capabilities

Fluorescence occurs after a molecule known as a chromophore absorbs photons of light. The molecule is raised to an excited state as a result of electron transfer from a ground state to a higher energy level. As the electrons drop back to the ground state they emit energy in the form of light or a quantum of light. **Excitation and Emission** described; is absorption and fluorescence of the molecule where the difference between these two energy states is the wavelength maxima or peak. For instructional purposes, wild type GFP has an excitation peak at 395 nm liberating an emission peak at 508 nm. The absorption intensity of the molecule is the **extinction coefficient** (probability of absorption). The emission intensity directly relates to the **quantum yield**; which is the ratio of the light released to light absorbed. Taken together the product of the extinction coefficient and the quantum yield result in the **fluorescence intensity**.

GFP wild type has an extinction coefficient of 27,600 $cm^{-1}M^{-1}$ and a quantum yield between 0.75 and 0.85. Compared to fluorescein and its extinction coefficient of 79,000 $cm^{-1}M^{-1}$ and a quantum yield of 0.9, GFP is less bright fluorescein. Compared to antibody labeling, however; GFP is more resistant to photo bleaching and avoiding background caused by non-specific binding of primary and secondary antibodies to targets other than the antigen. Although binding of multiple antibody to a single target offers a potential amplification not available for GFP, this is offset because neither labeling of the antibody nor binding of the antibody to the target is 10% efficient.

Effects of pH

Wild-type GFP at high pH (11-12) loses absorbance and excitation amplitude at 395 nm and gains amplitude at 470 nm. Wild-type GFP is also quenched by acidic pH values with an apparent pK near 4.5. Several of the mutants with enhanced spectral properties at pH 7 are actually more acid sensitive than is the wild type: EGFP is 50% quenched at pH 5.5. Many of the yellow fluorescent protein mutants, S65G, S72A, V68L, Q69K,T203Y, and the “TOPAZ” mutants exhibit pK’s as high as 6.8. The mechanistic explanation for these relatively high pKs is not entirely clear but may be due to the inherent stability in the stacked pi-electron system of the chromophore. The effect of acid is to quench the fluorescence altogether rather than simply shift it toward the short wavelengths expected of a protonated chromophore. The sensitivity of some GFPs to mildly acidic pH values carries both advantages and disadvantages. Such GFPs could be quenched to a major extent in acidic organelles such as lysosomes, endosomes, and Golgi compartments. The pH sensitivity of some GFPs can also be put to good use to measure organelle pH by targeting appropriate GFPs to those locations.

Effects of Temperature and Protein Concentrations

Higher GFP concentrations amplify the main excitation peak at 395 nm at the expense of the subsidiary peak at 470 nm. Increasing temperature from 15 to 65°C modestly decreases the 395-nm and increases the 470-nm excitation peak of mature wild-type GFP. Yet higher temperatures cause denaturation, with 50% of fluorescence lost at 78°C. More modest temperature increases from 20 to 37°C can profoundly decrease maturation efficiency of GFPs lacking mutations to improve folding.

Effects of Prior Illumination

GFPs have a variety of remarkable abilities to undergo photochemical transformations, which enables visualization of the diffusion or trafficking of GFP-tagged proteins. A defined zone within a cell or tissue is momentarily exposed to very bright illumination, which initiates the photochemistry. The subsequent fate of the photoconverted protein is imaged over time. At least four distinct types of semi-permanent photochemical transformation have been reported from one or more GFPs:

(a) Simple irreversible photobleaching

(b) Conversion from a 395 to 475-nm excitation maximum

(c) Loss of 488-nm-excited fluorescence, reversible by illumination at 406 nm

(d) Generation of rhodamine-like orange or red fluorescence upon illumination at 488 nm under strictly anaerobic conditions.

Applications

There are many applications currently utilizing GFP and its variants.

Experimentally, GFP emits bright green fluorescence when illuminated by blue or UV light. GFP Fluorescence is species-independent and does not require cofactors, substrates or additional gene products from *Aequorea victoria*. GFP can also be imaged in living samples making possible real time analysis of molecular events. GFP can be used to report protein fusion events with other proteins, in vivo localization of fusion proteins, and protein glycosylation events. Variants of GFP are instrumental as Fluorescence resonance energy transfer (FRET) reporter proteins. FRET is a process whereby one fluorophore transfers excitation energy to a neighboring fluorophore by overlapping the emission spectrum of the donor molecule with the excitation spectrum of the accepting molecule. This is a useful method of studying protein-protein interactions. Other uses include changes in gene expression, visualization of pathways in real time, targeting of subcellular structures, viral pathways, and proteomics.

Examples

Some examples of images involving different examples of GFP acquired with our systems (*Figures 3 and 4*). Both of these images are pseudo-colored displaying what is actually seen in the EpiChemi II Darkroom through our Fluorescent Sample Viewer.

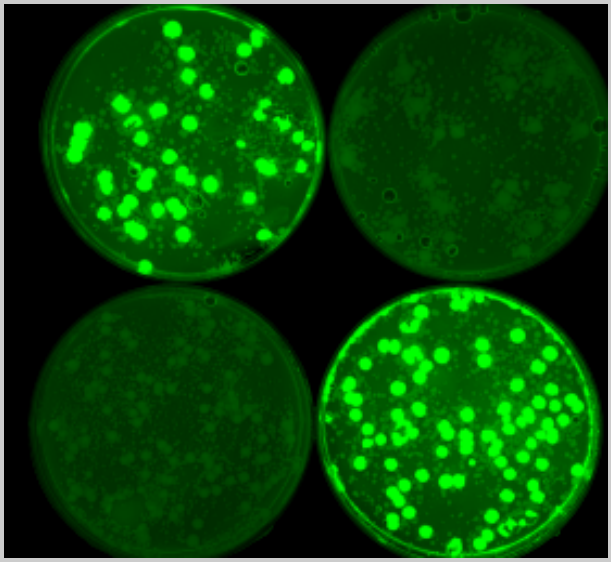

Figure 3. Four plates of E. coli k12 HB101 cells. Two have been expressed with GFP originating from pGLO™ Transformation system under antibiotic selection with two non-expressing controls. The transformed E. Coli and control plates were incubated 72 hours at 37°C. This image was acquired using UVP's BioChemi System with epi-illumination at a wavelength of 365nm for GFP excitation. For a strong contrast the Ethidium Bromide interference filter of the EpiChemi II Darkroom was used. An integration time of 5 – 8 seconds was used.

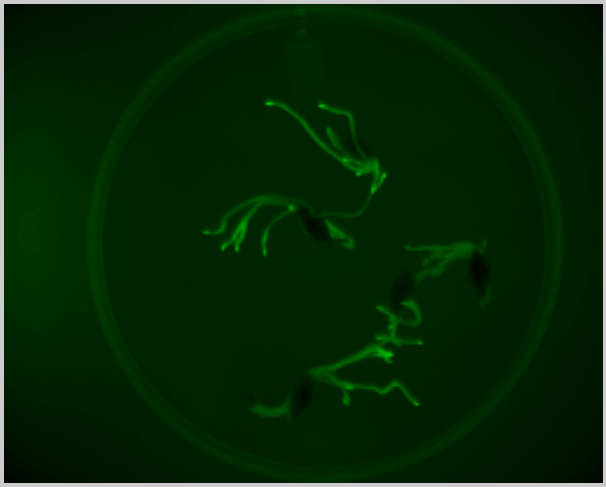

Figure 4. A barley seed had been imaged in a petri dish and documented for future analysis using LabWorks™ Software. This image was acquired at Guelph University in Western Ontario with UVP's BioChemi™ System using 480nm visi-blue transillumination and the green interference filter of the EpiChemi™ II Darkroom. The researcher was experimenting with maximizing growth rates and yields of a new variety of seed and wanted to quantify the expression of the root ends. Using the densitometry tools in LabWorks the root ends were defined and quantitated.

References

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3. Alice Y. Ting, Kristin H. Kain, Richard L. Klemke, Roger Y. Tsien; *Genetically encoded fluorescent reporters of protein tyrosine kinase acivities in living cells*; proceedings of the National Academy of Sciences; Dec. 2001; 98(26):15003-15008
4. Geoffrey S. Baird, David A. Zacharias, Roger Y. Tsien*; Circular permutation and receptor insertion within green fluorescent proteins*; Proceedings of the National Academy of Sciences; Jul. 1999; 96:11241-11246
5. A.B. Cubitt, R. Heim, S.R. Adams, A.E. Boyd, L.A. Gross, R.Y. Tsien *Understanding, improving and using green fluorescent proteins*; Trends in Biochemical Sciences. Nov. 1995; 20(11):448-55
6. Special thanks is extended to BIO-RAD's Biotechnology Explorer Program, BIO-RAD Laboratories, 2000 Alfred Nobel Drive, Hercules, CA 94547
7. CLONTECH – Living Colors™ Fluorescent Proteins and Living Colors red Fluorescent Proteins; 1020 East Meadow Circle, Palo Alto, CA 94303-4230

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